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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGBx EFFECT

III. THE INTERACTION BETWEEN PGB_x AND RAT LIVER MITOCHONDRIA

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Tritium labelled PGB_X-active oligomers were synthesized by alkaline polymerization of ^3H-PGB_1. When added to PGB_X assay system non-stoichiometric amount of ^3H-PGB_{1X} were bound to RLM. The degree of binding was dependent upon the concentration of ^3H-PGB_{1X} in the reaction. On the assumption that ^3H-PGB_{1X} and PGB_X are similar or identical in structure, then no correlation between PGB_X effect and PGB_X binding by RLM could be found.

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INTRODUCTION

The therapeutic use of PGB_X (1, 2, 3) in the treatment of human ischemic pathologies was suggested by successful animal experiments in a number of laboratories (4, 5, 6, 7, 8, 9, 10). Before human trials maybe attempted, it is necessary to first know the mechanism of the in vivo action of PGB_X. Towards this end studies have been underway in this laboratory to elucidate the in vitro mechanism of the PGB_X effect on phosphorylation activity of degraded RLM, in the hope that this would then lead to an understanding of the in vivo mechanisms of action.

In an earlier report (11) it was shown that the sequence in which PGB_X was added to the in vitro PGB_X assay system was important. When PGB_X was added to already degraded RLM, no maintenance of high phosphorylation activity was observed. Only when RLM were degraded in the presence of PGB_X could the PGB_X effect be demonstrated. These results suggested that RLM reacts with PGB_X to form a complex that protects the RLM from exposure to the hypotonic degradation medium. In this paper more direct evidence for this interaction is reported.

METHODS AND MATERIALS

PGB_1-methyl ester, 15-keto PGB_1-methyl ester and PGB_X Type II were synthesized as described previously (2, 3). ^3H-PGB_1, labelled in the 5, 6 position, was purchased from New England Nuclear Corporation (Boston, MA). Radioactivity was measured with a Beckman Model LS-200B Liquid Scintillation Syst (Fullerton, CA) using "Bray's Mixture" (12) (0.2g dimethyl POPOP, 4g PPO, 60g napthalene, 100 ml methanol, 20 ml ethylene glycol, and dioxane to make one liter). RLM phosphorylation activity was measured as previously reported (2, 3).

Note: Abbreviations used in this report are: RLM, rat liver mitochondria; Pi, inorganic phosphate; PG-, prostaglandin; PGB_X, oligomeric mixture from alkaline polymerization of 15-keto PGB_1; PGB_{1X}, oligomeric mixture from alkaline polymerization of PGB_1; PPO, diphenyloxazole; dimethyl POPOP, 1,4-BIS (2-(4-methyl-5-phenyloxazoly l)) benzene

EXPERIMENTAL

The best method for the measurement of the PGB_x-RLM complex entails the separation of the RLM from the remainder of the reaction medium and then the analysis of the RLM for its PGB_x content. The separation of the RLM is easily accomplished by centrifugation; the analysis of the PGB_x content however is not easily accomplished. At the present time, methods for the quantitative analysis for PGB_x is based on its characteristic UV absorption or fluorescence spectra. However, these optical measurements cannot be used with RLM directly because of their insolubility. An alternative procedure then would be to analyze the clear supernatant solutions from reaction media in which RLM was first sedimented. Any decrease in the soluble PGB_x content from that amount of PGB_x added would then be a measure of the PGB_x bound to RLM. Attempts to use the optical methods for PGB_x analysis of the clear supernatant solutions were not successful, because the constituents of the assay medium also exhibited similar UV absorption and fluorescence spectra, and thus interfered with the measurement of trace amounts of PGB_x.

It appeared obvious then, that the successful determination of PGB_x bound to RLM would require a very sensitive and specific analytical method. It is well known that the use of radioactive tracers permits the specific analysis of trace amounts of material, and this method then appeared to be the method of choice for this study.

Synthesis of ^3H-PGB_{1x}: The synthesis of radioactive labelled PGB_x from 15-keto PGB_1 was not feasible because of commercial unavailability. Since ^3H-PGB_1 is available commercially, and since PGB_1 had been converted to active PGB_x by Polis et al (1) it was decided to prepare an oligomer of ^3H-PGB_1 that would exhibit in vitro PGB_x properties. This oligomeric mixture was designated ^3H-PGB_{1x} to show the identity of the precursor used. ^3H-PGB_{1x} was synthesized by dissolving a mixture of 50μC of ^3H-PGB_1 and 10μg of PGB_1-methyl ester in 0.4 ml of ethanolic-KOH (50% ethanol - 1.0N KOH) and heating the mixture in a stoppered flask at 50° for five hours. The polymerization reaction was stopped by adding 10 ml of H_2O and acidifying pH 3.0 with HCl. The PGB_x synthetic conditions used in this study were found to be optimal from preliminary test polymerizations of unlabelled PGB_1-methyl ester in which the time and temperature of the reaction were varied. ^3H-PGB_{1x} was purified by extraction into ethyl acetate (2 extractions, 20 ml each), washing the combined ethyl acetate layers with H_2O (4 times with, 15 ml) to remove excess acid, and centrifugation to remove insoluble material. The solvent was removed by flash-evaporation at 50° and the residue dried to constant weight. Yield: 9.1mg (91%); specific

activity, 3194cpm/µg. For use in this study, the ^{3}H-PGB$_{1x}$ was dissolved in 25% ethanol-0.0025M $NaHCO_3$ to a final concentration of 10mg/ml and stored at 0° until used.

^{3}H-PGB$_{1x}$ was assayed for the in vitro PGB$_x$ effect as described previously (2, 3) over a concentration range of 1.36 to 36.36µg per ml of reaction medium. These results and the results of the assay of PGB$_x$ Type II are shown in Figure 1. The PGB$_x$ activity of ^{3}H-PGB$_{1x}$ was almost equivalent to that of PGB$_x$ Type II except that at the higher concentration, ^{3}H-PGB$_{1x}$ exhibited less inhibitor effect.

Binding of ^{3}H-PGB$_{1x}$ by RLM: To four beakers containing the reagents of the degradation mixture of the PGB$_x$ assay system (2, 3), aliquots of stock ^{3}H-PGB$_{1x}$ were added so that each beaker contained either 1 to 15µg/ml reaction. After equilibration at 27°, 4mg of RLM, aged four days at 0° in 0.3M sucrose, were added and the mixture incubated at 27° for eight minutes. At the end of this time period, the mixture was centrifuged at 8000g for ten minutes to sediment the RLM. The radioactivity in the supernatant was determined by adding 0.1 ml of clear supernatant to 10 ml of Bray's scintillation cocktail and counting the radioactivity for at least two minutes. In another identical series of tests, the nucleotide mixture of stage 2 of the PGB$_x$ assay system was added and the mixture incubated for 20 minutes. At the end of this time the mixture was centrifuged at 8000g for ten minutes and aliquots were removed for radioactivity counting and in addition, another aliquot was deproteinized and the phosphorylation activity determined as described previously (2, 3). The phosphorylation results are plotted in Figure 1. The amount of ^{3}H-PGB$_{1x}$ in the soluble fraction of each reaction mixture was calculated using the specific activity of ^{3}H-PGB$_{1x}$. By subtracting the soluble ^{3}H-PGB$_{1x}$ from the total ^{3}H-PGB$_{1x}$ added, the amount of ^{3}H-PGB$_{1x}$ sedimenting with the RLM was calculated. Figure 2 shows the plot of ^{3}H-PGB$_{1x}$ bound to RLM in terms of µg/mg RLM as a function of total ^{3}H-PGB$_{1x}$ added. The solid line represents the degradation stage only, while the dashed line represents the total PGB$_x$ assay system. Although the phosphorylation reactions appear to cause a discharge of bound ^{3}H-PGB$_{1x}$ as evidenced by the lower amounts of ^{3}H-PGB$_{1x}$ bound, the overall picture actually suggests no change in bound ^{3}H-PGB$_{1x}$ and the lowered results maybe experimental error. The amount of ^{3}H-PGB$_{1x}$ bound to RLM appears to be dependent upon the amount of ^{3}H-PGB$_{1x}$ added to the system. The maximum PGB$_x$ effect takes place at the level between 10-20µg ^{3}H-PGB$_{1x}$. At higher levels, where ^{3}H-PGB$_{1x}$ decreases phosphorylation, a greater amount is bound to the RLM.

DISCUSSION

On the assumption that the PGB_x-active oligomers formed during the alkaline polymerization of PGB_1 (PGB_{1x}) are similar or identical to PGB_x oligomers, then the binding of PGB_{1x} by RLM also means the RLM binds PGB_x in a similar fashion. On this basis then, RLM binds non-stoichiometric amounts of PGB_x and that the degree of binding is dependent upon the concentration of PGB_x added. As to whether the RLM-PGB_x complex is important in the PGB_x effect on RLM oxidative phosphorylation, is not answered by the results of this study. The results, however, do show that a correlation does not exist between the PGB_x effect and the degree of PGB_x binding to RLM, since at low PGB_x: RLM proportions, the phosphorylating activity is high; at high PGB_x: RLM proportions, the phosphorylating activity is low.

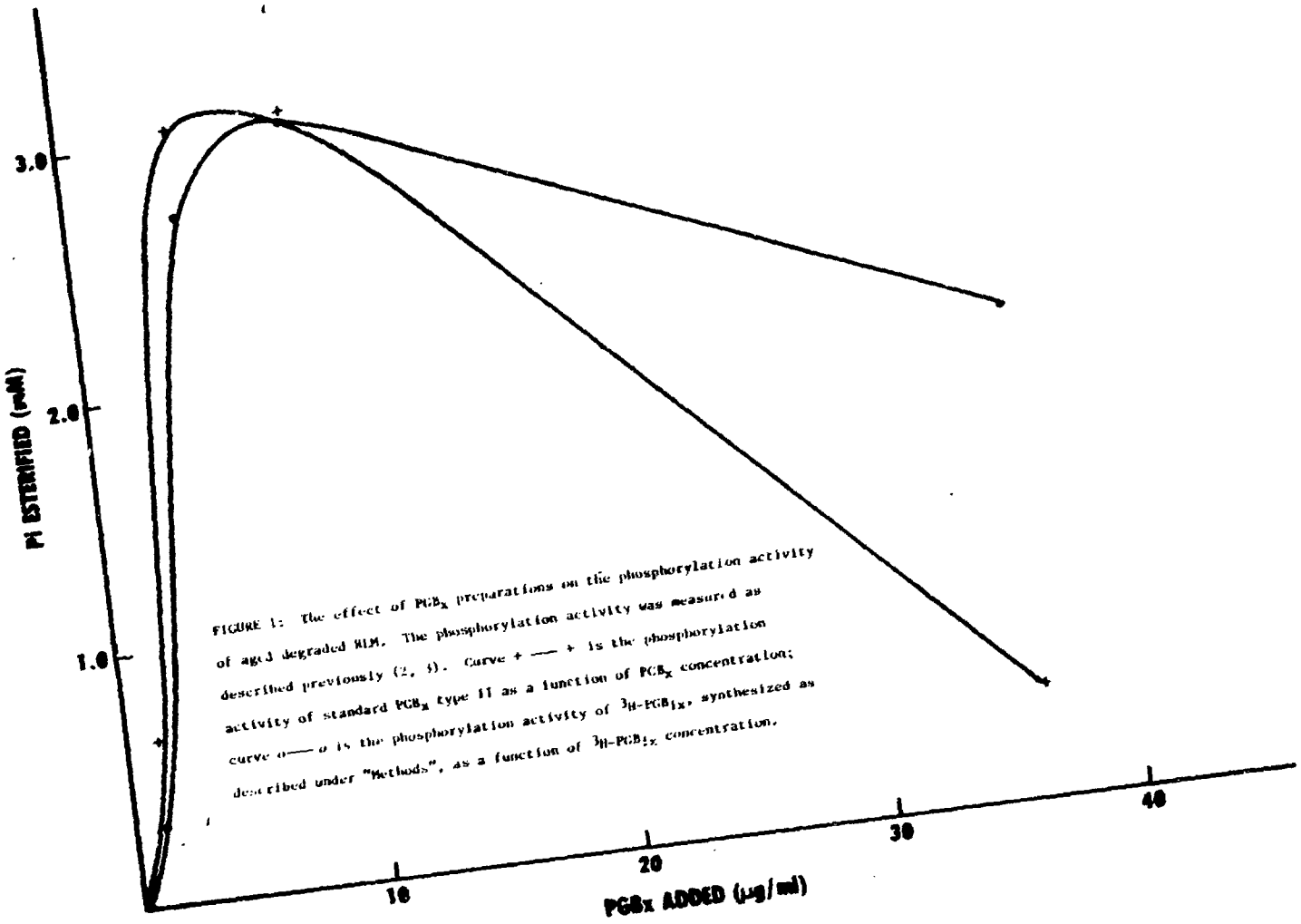

FIGURE 1: The effect of PGB_x preparations on the phosphorylation activity of aged degraded RIM. The phosphorylation activity was measured as described previously (2, 3). Curve + —— + is the phosphorylation activity of standard PGB_x type II as a function of PGB_x concentration; curve o—— o is the phosphorylation activity of ^3H-PGB_{1x}, synthesized as described under "Methods", as a function of ^3H-PGB_{1x} concentration.

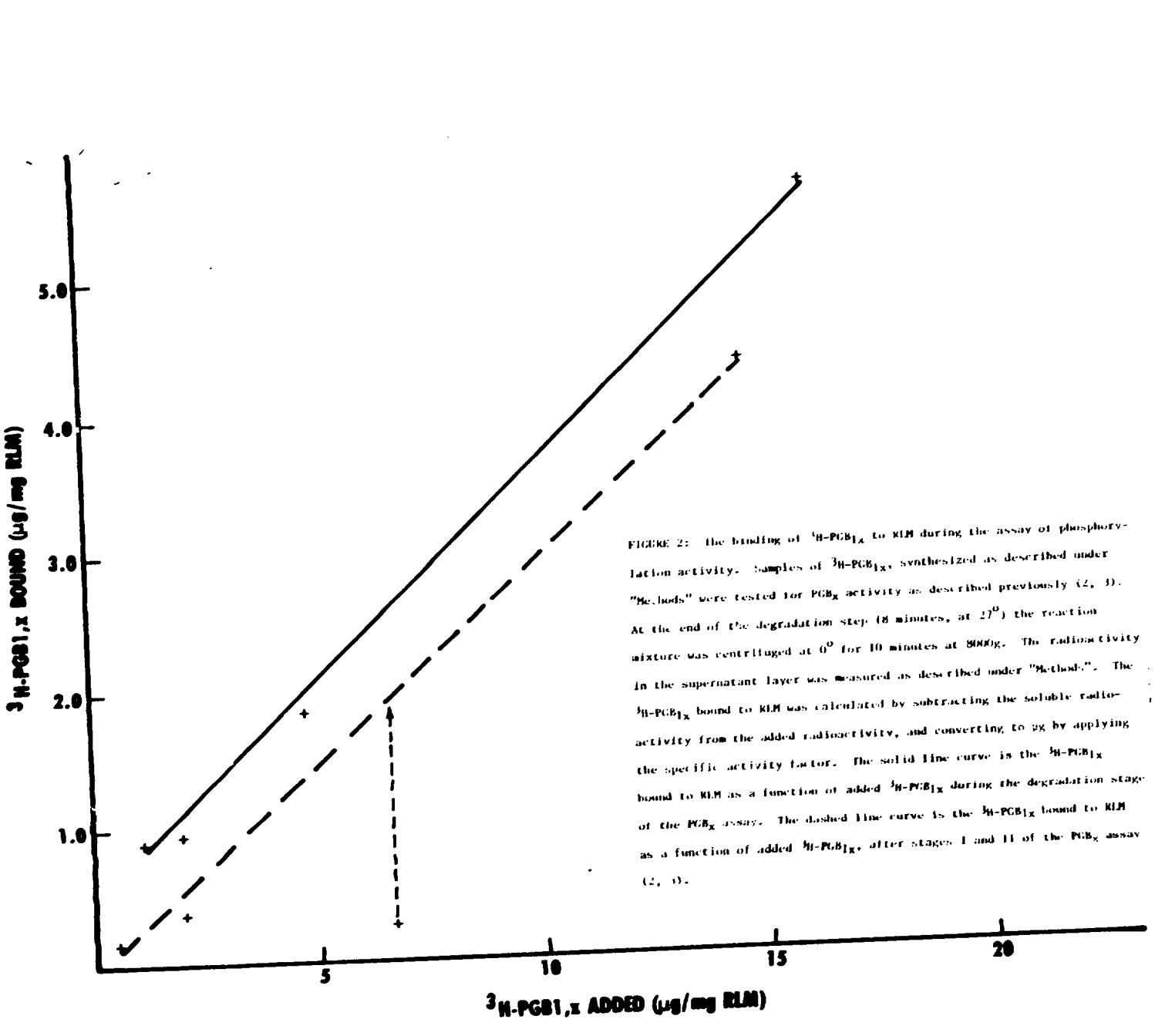

FIGURE 2: The binding of ^{3}H-PGB_{1x} to RLM during the assay of phosphorylation activity. Samples of ^{3}H-PGB_{1x}, synthesized as described under "Methods" were tested for PGB_x activity as described previously (2, 3). At the end of the degradation step (8 minutes, at 27°) the reaction mixture was centrifuged at 0° for 10 minutes at 8000g. The radioactivity in the supernatant layer was measured as described under "Methods". The ^{3}H-PGB_{1x} bound to RLM was calculated by subtracting the soluble radioactivity from the added radioactivity, and converting to µg by applying the specific activity factor. The solid line curve is the ^{3}H-PGB_{1x} bound to RLM as a function of added ^{3}H-PGB_{1x} during the degradation stage of the PGB_x assay. The dashed line curve is the ^{3}H-PGB_{1x} bound to RLM as a function of added ^{3}H-PGB_{1x}, after stages I and II of the PGB_x assay (2, 3).

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